In Vitro Interactions between Amphotericin B, Itraconazole, and Flucytosine against 21 Clinical *Aspergillus* Isolates Determined by Two Drug Interaction Models

D. T. A. Te Dorsthorst,[1,2,3] P. E. Verweij,[2,3] J. F. G. M. Meis,[1] N. C. Punt,[4] and J. W. Mouton[1]*

Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital Nijmegen,[1] Department of Medical Microbiology, University Medical Center Nijmegen,[2] and Nijmegen University Center for Infectious Diseases,[3] Nijmegen, and Medimatics, Maastricht,[4] The Netherlands

Combination therapy of flucytosine (5FC) with other antifungal agents could be of use for the treatment of invasive aspergillosis. However, interpretation of the results of in vitro interactions is problematic. The fractional inhibitory concentration (FIC) index is the most commonly used method, but it has several major drawbacks in characterizing antifungal drug interaction. Alternatively, a response surface approach using the concentration-effect relationship over the whole concentration range instead of just the MIC can be used. We determined the in vitro interactions between amphotericin B (AMB), itraconazole, and 5FC against 21 *Aspergillus* isolates with a broth microdilution checkerboard method that employs the dye MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide]. FIC indices based on three different MIC endpoints (MIC-0, MIC-1, and MIC-2) and the interaction coefficient alpha were determined, the latter by estimation from the response surface approach described by Greco et al. (W. R. Greco, G. Bravo, and J. C. Parsons, Pharmacol. Rev. 47:331–385, 1995). The value obtained for the FIC index was found to be dependent on the MIC endpoint used and could be either synergistic, indifferent, or antagonistic. The response surface approach gave more consistent results. Of the three combinations tested, the AMB-5FC combination was the most potent in vitro against *Aspergillus* spp. We conclude that the use of the response surface approach for the interpretation of in vitro interaction studies of antifungals may be helpful in order to predict the nature and intensity of the drug interaction. However, the correlation of these results with clinical outcome remains difficult and needs to be further investigated.

In the past 2 decades, the incidence of invasive aspergillosis has increased. This increase is directly related to the growing number of people at risk because of solid organ transplantation, hematopoietic stem cell transplantation, and intensified chemotherapeutic regimens. Invasive aspergillosis is usually caused by *Aspergillus fumigatus* and to lesser extent by *Aspergillus flavus*, *Aspergillus niger*, and *Aspergillus terreus* (8, 18). The morbidity and mortality of invasive aspergillosis remain high.

Amphotericin B (AMB) and itraconazole (ITZ) were the only two antifungal agents available for the treatment of invasive aspergillosis for a long period of time, but recently voriconazole was shown to be superior to AMB and other licensed antifungal therapies for first-line treatment of invasive aspergillosis (11). Treatment with AMB is associated with nephrotoxicity, and resistance to AMB has been reported for *A. fumigatus* (31), *A. flavus* (26), and *A. terreus* (3). The resistance of *A. fumigatus* to ITZ, both in vitro and in vivo, has been described previously (4, 6), and cross-resistance to antifungal azoles was described for a clinical *A. fumigatus* isolate; MICs of ITZ and voriconazole and of the investigational azoles ravuconazole and posaconazole for this isolate were high (32).

An alternative approach for the treatment of invasive aspergillosis could be the use of combination therapy. Combination therapy is successfully used for difficult-to-treat bacterial infections, including mycobacterial infections (12), and viral infections, including human immunodeficiency virus infection (http://www.aidsinfo.nih.gov). Possible advantages of combination therapy are the improvement of the efficacy of antifungal therapy due to synergistic drug interaction, the reduction of drug levels required to produce the antifungal effect and consequently the reduction of side effects, reduced risk for the development of resistance, and possibly a shorter duration of therapy (27). Of course, there are also possible disadvantages of combination therapy, such as toxicity, negative drug interaction of the two drugs with each other or with other drugs, and cost.

Flucytosine (5FC) is one of the oldest antifungal agents. It was first synthesized in 1957 as a potential cytostatic agent. However, it was ineffective against tumors but was found to exert antifungal activity. The use of 5FC as a single agent is limited to the treatment of cases of chromoblastomycosis, uncomplicated lower urinary tract candidiasis, and vaginal candidiasis due to the pretreatment occurrence of resistant strains (about 10% of *Candida albicans* isolates) and the development of resistance of many fungi during monotherapy. In the vast majority of cases, 5FC is used concomitantly with other agents, mainly AMB, for the treatment of systemic mycosis, such as cryptococcosis, candidiasis, and aspergillosis. However, there is no evidence that this combination is more effective than AMB monotherapy in the treatment of both invasive candidal infections and invasive aspergillosis (29).

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* Corresponding author. Mailing address: Department of Medical Microbiology and Infectious Diseases, Canisius Wilhelmina Hospital, Weg door Jonkerbos 100, 6532 SZ Nijmegen, The Netherlands. Phone: 31-(0)24-3657514. Fax: 31-(0)24-3657516. E-mail: mouton@cwz.nl.

In order to predict the nature as well as the intensity of the drug interaction in clinical practice, in vitro susceptibility tests such as checkerboard studies may be helpful, although results of in vivo interactions, such as the toxicity of combined drugs, cannot be determined. In addition, the interpretation of in vitro interaction studies may be difficult (19). The concentration-effect relationship of antifungals extends over several two-fold dilutions, instead of growth versus no growth, as is the case for most antimicrobials. Consequently, the concentration at which the MIC is read is arbitrary. The value and interpretation of the fractional inhibitory concentration (FIC) index suffer from the same drawbacks. To overcome these disadvantages, a surface response approach may be used. In that approach, the whole interaction surface, i.e., all drug effects either alone or in combination and not just the MIC effect, is used to fit a model, and conclusions are drawn with respect to a positive or negative interaction.

In this study we determined the in vitro interactions between AMB and ITZ, AMB and 5FC, and ITZ and 5FC against 21 clinical *Aspergillus* isolates.

(Results of this investigation were partly presented at the 42nd Interscience Conference on Antimicrobial Agents and Chemotherapy, San Diego, Calif., 2002.)

MATERIALS AND METHODS

Isolates. Twenty-one clinical *Aspergillus* isolates were tested: five ITZ-susceptible (ITZ-S) *A. fumigatus* isolates (AZN 5161, AZN 7151, AZN 7319, AZN 7820, and AZN 8248), five ITZ-resistant (ITZ-R) *A. fumigatus* isolates (AZN 58, AZN 59, AZN 5241, AZN 5242, and AZG 7), five *A. flavus* isolates (AZN 510, AZN 6578, AZN 6686, AZN 6803, and AZN 6837), and six *A. terreus* isolates (AZN 3a, AZN 10, AZN 18, AZN 27, AZN 30, and AZN 31). The isolates with the AZN prefix were obtained from the private collection of the Department of Medical Microbiology, University Medical Center Nijmegen, Nijmegen, The Netherlands, and isolate AZG 7 was obtained from the University Hospital Groningen, Groningen, The Netherlands (30). Isolates had been frozen in glycerol broth at −80°C and were revived by subculturing them on Sabouraud glucose agar tubes supplemented with 0.5% chloramphenicol and incubating them for 5 to 7 days at 35°C. They were subcultured again on Sabouraud glucose agar for 5 to 7 days at 35°C before preparation of the inoculum. All isolates were tested in triplicate on different days.

Candida parapsilosis (ATCC 22019) and *Candida krusei* (ATCC 6258) were used for quality control in all experiments.

Antifungal agents. AMB (Bristol-Myers Squibb, Woerden, The Netherlands), ITZ (Janssen Pharmaceutica B.V., Tilburg, The Netherlands), and 5FC (ICN Pharmaceuticals, Zoetermeer, The Netherlands) were obtained as powders. AMB and ITZ were dissolved in dimethyl sulfoxide, and 5FC was dissolved in distilled water to make stock solutions. The stock solutions were held at −80°C until use. Serial twofold dilutions of each antifungal agent were prepared according to NCCLS M38-A guidelines (23). Final dilutions were made in RPMI 1640 medium (with L-glutamine, without bicarbonate) (GIBCO BRL, Life Technologies, Woerden, The Netherlands) buffered to pH 7.0 with 0.165 mol of 3-[*N*-morpholino]propanesulfonic acid (MOPS) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) per liter.

Susceptibility testing. MICs were determined by a modified broth microdilution method that employs the dye MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] (20). The final concentrations of all antifungal agents ranged from 0.016 to 16 µg/ml. Because off-scale MICs were found for 5FC against the *A. fumigatus* and the *A. terreus* isolates, higher concentrations (0.25 to 256 µg/ml) were tested. Aliquots of 100 µl of the drugs at a concentration of two times the targeted final concentration were dispensed in the wells of flat-bottom 96-well microtiter plates (Costar, Corning, N.Y.).

Conidial suspensions were prepared spectrophotometrically (23) and were further diluted in RPMI 1640 medium containing MTT (0.2 mg/ml; Sigma Chemical Co., St. Louis, Mo.). In order to obtain a final inoculum concentration of 0.4×10^4 to 5×10^4 CFU/ml and a final MTT concentration of 0.1 mg/ml (at this concentration MTT has no effect on the growth of the fungi [20]), 100 µl of

the inoculum was added to the wells. The microtiter plates were incubated for 48 h at 35°C.

After 48 h, the content of each well was removed, and 200 µl of isopropanol containing 5% HCl (1 N) was added to extract the dye. After 30 min of incubation at room temperature and gentle agitation, the optical density at 540 nm (OD_{540}) was measured with a microplate reader (Anthos htIII; Anthos Labtec Instruments, Salzburg, Austria). The ODs of the blank wells were subtracted from the ODs of the inoculated wells.

The percentage of MTT conversion to its formazan derivate for each well was calculated by comparing the OD_{540} of the well with that of the drug-free control based on the following equation: (OD_{540} of the well that contained the drug/ OD_{540} of the drug-free well) $\times$ 100%.

For AMB, the lowest concentration that showed 10% or less of the growth of the growth control (MIC-0) was taken as MIC endpoint (21). For ITZ and 5FC, the lowest concentration that showed 50% or less of the growth of the growth control (MIC-2) was taken as MIC endpoint.

Interaction of drugs in vitro. Drug interactions were assessed by a modified checkerboard broth microdilution method that employs the dye MTT. The final concentration of the antifungal agents ranged from 0.016 to 16 µg/ml for AMB, from 0.008 to 8 µg/ml for ITZ, and from 0.062 to 64 µg/ml for 5FC.

Aliquots of 50 µl of each drug (and in the case of the single-drug control, 50 µl of that drug and 50 µl of medium with the drug solvent at the final concentration) at a concentration four times the targeted final concentration were dispensed in the wells in order to obtain a two-dimensional checkerboard.

Conidial suspensions were prepared as described above, and 100 µl of the inoculum was added to the wells. The microtiter plates were incubated for 48 h at 35°C. After 48 h, the conversion of MTT was measured as described above.

Drug interaction modeling. The data obtained were analyzed using two different models. The models were nonparametric and parametric approaches of the Loewe additivity zero-interaction theory.

FIC index model. The FIC index, which is the nonparametric approach, was defined as follows (22): $\Sigma FIC = FIC_A + FIC_B$ = (MIC of drug A in combination/ MIC of drug A alone) + (MIC of drug B in combination/MIC of drug B alone).

Since each triplicate experiment yielded several ΣFICs, among all ΣFICs calculated for each replicate, the minimum ΣFIC (ΣFIC_{min}) and maximum ΣFIC (ΣFIC_{max}) were determined to correspond to the lowest and the highest ΣFIC, respectively. The reported FIC index was the ΣFIC_{min} in all cases unless the ΣFIC_{max} was higher than 4, and then the ΣFIC_{max} was reported as the FIC index. In order to determine the larger departure from additivity, the ΣFIC_{max} was also reported as the FIC index for data sets where the ΣFIC_{max} was smaller than 4 but the ΣFIC_{min} was greater than 1. If for a data set the ΣFIC_{min} was lower than 0.5 and the ΣFIC_{max} was higher than 4, both the ΣFIC_{min} and the ΣFIC_{max} were reported.

The three MIC endpoints used, namely, MIC-0, MIC-1, and MIC-2, were defined as the lowest drug concentrations showing no more than 10, 25, and 50% of the growth of the growth control, respectively. Thus, the following FIC indices were determined for each replicate: FIC-0, FIC-1, and FIC-2, respectively. Off-scale MICs were converted to the next-higher or -lower twofold concentration. Since the FIC index is not normally distributed, the median and the range of the FIC indices among the replicates were calculated. For the interpretation of the results obtained with the FIC index, we considered the results of all three replicates as one outcome. Thus, when the FIC indices from all three replicates were smaller than 1, synergism was claimed. When the FIC indices from all three replicates were higher than 1, antagonism was claimed, and in all other cases, indifference was concluded.

Greco model. The fully parametric surface approach described by Greco et al. (9) was based on the following equation:

$$1 = \frac{D_A}{IC_{50,A}\left(\dfrac{E}{E_{con} - E}\right)^{1/m_A}} + \frac{D_B}{IC_{50,B}\left(\dfrac{E}{E_{con} - E}\right)^{1/m_B}}$$
$$+ \alpha \frac{D_A D_B}{IC_{50,A} IC_{50,B}\left(\dfrac{E}{E_{con} - E}\right)^{0.5(1/m_A + 1/m_B)}}$$

where E is the percentage of growth (dependent variable) at the drug concentrations D_A and D_B (independent variables), E_{con} is the maximal percentage of growth observed in the drug-free control, $IC_{50,A}$ and $IC_{50,B}$ are the inhibitory concentrations of drug A and drug B producing 50% of the E_{con}, m_A and m_B are the slopes of the concentration-effect curves (Hill coefficient) for drug A and drug B, and α is the interaction parameter (ICα) which describes the nature of the interaction. This model was fitted directly to the entire data set (percentages

TABLE 1. Results of the susceptibility testing of AMB, ITZ, and 5FC against several *Aspergillus* isolates

Species (no. of isolates)	MIC$_{50}$ (µg/ml) (range)[a]		
	AMB	ITZ	5FC
ITZ-S *A. fumigatus* (5)	0.25 (0.25)	0.125 (<0.016–0.25)	8 (2–128)
ITZ-R *A. fumigatus* (5)	0.25 (0.125–1)	4 (0.5–>16)	16 (2–>256)
A. flavus (5)	0.5 (0.25–0.5)	0.125 (<0.016–0.25)	2 (0.25–4)
A. terreus (6)	1 (0.5–2)	0.031 (<0.016–0.062)	1.5 (<0.25–>256)

[a] MIC$_{50}$ and range are shown for three replicate experiments. For AMB, MIC-0 was used. For ITZ and 5FC, MIC-2 was used.

of growth for all concentrations of the two drugs alone or in combination) by using a nonweighted, nonlinear regression analysis and a computer program (ModLab; Medimatics, Maastricht, The Netherlands). The program estimates the model parameters, and it also calculates the 95% confidence interval (95% CI) for each parameter. Goodness-of-fit criteria included the R^2. Obtained parameters were validated using the program Syner, kindly provided to us by G. L. Drusano (Albany Medical College, Albany, N.Y.).

The median and the range of the ICα among the replicates were calculated. For the interpretation of the results obtained with the Greco model, we considered the results of all three replicates as one outcome. Thus, when the ICα was higher than 0 for all three replicates, synergism was claimed. When the ICα was lower than 0 for all three replicates, antagonism was claimed. In all other cases, indifference was concluded.

RESULTS

All *Aspergillus* isolates grew well after 48 h of incubation at 35°C. In each batch of broth microdilution tests, the MICs of the quality control strains were within the reference ranges.

MIC data. Table 1 shows the results of the susceptibility testing of AMB, ITZ, and 5FC against the *Aspergillus* isolates. The MICs of AMB ranged from 0.125 to 2 µg/ml, with the MIC at which 50% of the isolates tested were inhibited (MIC$_{50}$) being 0.5 µg/ml. The MICs of ITZ ranged from <0.016 to >16 µg/ml, with the MIC$_{50}$ being 0.125 µg/ml, and the MICs of 5FC ranged from <0.25 to >256 µg/ml, with the MIC$_{50}$ being 4 µg/ml.

In vitro interactions. Table 2 shows the results obtained with the FIC index model and the Greco model for each species-drug combination. The results, and sometimes also the inter-

actions, found by both the FIC index model and the Greco model depended on the species tested. Importantly, the results and conclusions drawn from the FIC index model depended primarily on the MIC endpoint used. The results of the various FIC indices were, however, not completely contradictory, in that for all data sets for which the FIC$_{min}$ was reported, the FIC$_{max}$ was lower than or equal to 2.5, and for all data sets for which the FIC$_{max}$ was reported, the FIC$_{min}$ was greater than or equal to 0.5. The difference between the FIC$_{min}$ and the FIC$_{max}$ never ranged from <0.5 to >4.

AMB-ITZ combination and the FIC index model. Depending on the definition of the FIC used (FIC-0, FIC-1, or FIC-2), a synergistic, indifferent, or antagonistic interaction was found with a significant portion of the isolates (Table 2). Both the FIC-0 and the FIC-1 for the *A. flavus* isolates were significantly higher ($P < 0.05$) than those for the ITZ-S *A. fumigatus*, the ITZ-R *A. fumigatus*, and the *A. terreus* isolates. The FIC-2 for the ITZ-R *A. fumigatus* isolates were significantly lower than those for the *A. flavus* and the ITZ-S *A. fumigatus* isolates, and the FIC-2 for the *A. terreus* isolates were significantly lower than those for the *A. flavus* isolates.

Greco model. Regarding the goodness of fit, the R^2s were above 0.90 in the majority of cases (median, 0.93; range, 0.07 to 0.98).

Antagonism was found with 33.3%, indifference was found with 57.2%, and synergism was found with 9.5% of all 21 isolates. The ICα values for the ITZ-R *A. fumigatus* isolates

TABLE 2. Summarized results of the two drug interaction models for the AMB-ITZ, AMB-5FC, and ITZ-5FC combinations for several *Aspergillus* isolates[c]

Drug combination	Species	Nonparametric result (FIC index)[a]			Parametric result (Greco ICα)[b]
		FIC-0	FIC-1	FIC-2	
AMB-ITZ	ITZ-S *A. fumigatus*	2.06 (0.53–2.50)	0.63 (0.53–2.25)	0.55 (0.09–0.63)	−0.02 (−0.04–0.01)
	ITZ-R *A. fumigatus*	2.50 (0.50–2.50)	0.56 (0.38–2.06)	0.25 (0.09–0.50)	1.05×10^2 (-1.57–1.50×10^6)
	A. flavus	4.13 (0.63–4.50)	2.25 (0.52–4.25)	0.53 (0.31–2.50)	−0.06 (−0.07–0.00)
	A. terreus	2.25 (0.53–4.25)	0.53 (0.52–2.25)	0.25 (0.19–1.50)	−0.01 (−0.07–0.58)
AMB-5FC	ITZ-S *A. fumigatus*	0.50 (0.50–1.00)	0.56 (0.38–1.00)	0.54 (0.19–1.00)	2.88 (-0.25–3.23×10^4)
	ITZ-R *A. fumigatus*	0.53 (0.50–1.00)	0.52 (0.50–2.00)	0.28 (0.09–0.75)	2.83 (0.00–17.49)
	A. flavus	0.51 (0.26–0.75)	0.56 (0.28–4.13)	0.63 (0.27–4.06)	0.09 (−0.08–3.54)
	A. terreus	2.01 (0.27–4.02)	0.22 (0.06–2.50)	0.31 (0.04–1.50)	2.20 (-2.06–2.34×10^{11})
ITZ-5FC	ITZ-S *A. fumigatus*	2.50 (1.00–8.50)	1.50 (0.50–2.50)	0.26 (0.16–2.06)	−0.21 (-7.97–1.69×10^{10})
	ITZ-R *A. fumigatus*	ND	ND	ND	8.21×10^2 (-1.00–2.49×10^{11})
	A. flavus	0.75 (0.25–2.25)	1.00 (0.31–2.50)	0.56 (0.19–2.50)	−0.04 (−0.09–2.09)
	A. terreus	4.50 (2.50–8.50)	2.50 (0.16–4.50)	0.44 (0.14–1.00)	−0.06 (-0.32–4.51×10^3)

[a] Three FIC indices were determined, namely, FIC-0, FIC-1, and FIC-2, using three different MIC endpoints. For ITZ-5FC, the FIC-0, FIC-1, and FIC-2 for the ITZ-R *A. fumigatus* isolates could not be determined due to full growth of the plate. ND, could not be determined.
[b] For the data sets in which the 95% CI did overlap 0, 0 was used as the ICα.
[c] The data are medians and ranges for three replicate experiments.
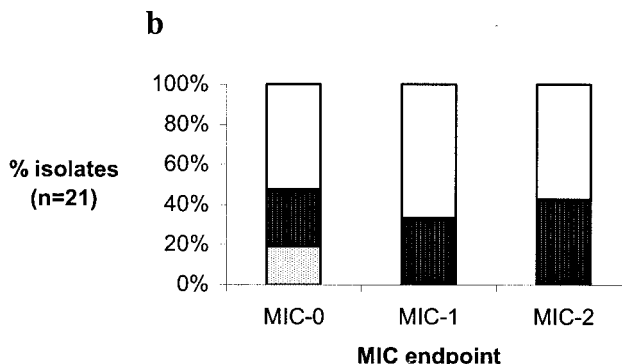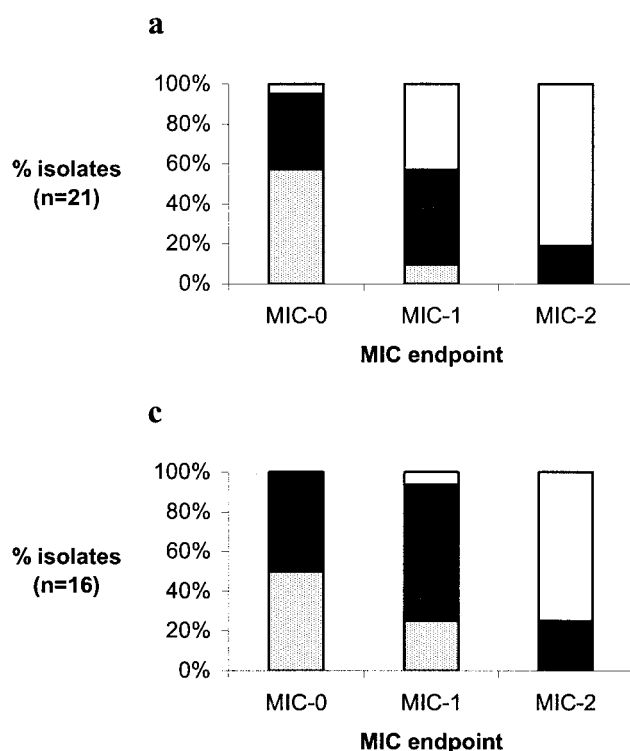
a

b

c

FIG. 1. Interactions found by using different MIC endpoints for the determination of the FIC index for the AMB-ITZ (a), the AMB-5FC (b), and the ITZ-5FC (c) combinations. Shaded, antagonism; black, indifference; white, synergism.

ences between the FIC-0 values for the ITZ-S *A. fumigatus*, the *A. flavus*, and the *A. terreus* isolates were significant. The highest FIC-0 values were found for the *A. terreus* isolates, followed by the ITZ-S *A. fumigatus* isolates and then by the *A. flavus* isolates. The FIC-1 values for the *A. flavus* isolates were significantly lower than those for the *A. terreus* isolates. The FIC-2 values for the *A. flavus* isolates were significantly higher than those for the *A. terreus* and the ITZ-S *A. fumigatus* isolates.

Greco model. The R^2 of all fits ranged from 0.40 to 0.95 (median, 0.83). Antagonism was found with 23.8%, indifference was found with 66.7%, and synergism was found with 9.5% of all 21 isolates. The ICα values for the ITZ-S *A. fumigatus* isolates were significantly lower than those for the ITZ-R *A. fumigatus*, the *A. flavus*, and the *A. terreus* isolates.

Effect of MIC endpoint on FIC index values. Since MICs of various drugs were determined at three endpoints, MIC-0, MIC-1, and MIC-2, FIC indices calculated were different depending on the endpoint used (FIC-0, FIC-1, and FIC-2). Figure 1 shows the interpretations resulting from the three different endpoints. It is clear from the figure that the value of the FIC index, and therefore the conclusions based thereon, is directly dependent on the endpoint used.

DISCUSSION

Determining the in vitro interaction between antifungals depends on the methodology used and certain variables, such as inoculum size and preparation, and on the approach used to analyze the results.

Previous studies in which the in vitro interactions between, for example, AMB and ITZ against *Aspergillus* species were determined yielded conflicting results (5, 15, 17, 28). This lack of agreement might be caused by differences in the methods used (agar dilution method, E-test, macrodilution method, and microdilution method) or by differences in variables such as inoculum size (which varies from 10^3 to 10^5 CFU/ml), ways of preparing the inoculum (hemacytometer or spectrophotometer), incubation times (24 to 48 h), incubation temperatures (30 to 37°C), and media (yeast nitrogen base or RPMI 1640). In this study we used a colorimetric broth microdilution checkerboard method, based on the conversion of the tetrazolium salt MTT to its formazan derivative, to determine the in vitro

were significantly higher than those for the *A. flavus*, the ITZ-S *A. fumigatus*, and the *A. terreus* isolates, and the ICα values for the *A. terreus* and the ITZ-S *A. fumigatus* isolates were significantly higher than those for the *A. flavus* isolates.

AMB-5FC combination and the FIC index model. Depending on the definition of the FIC used (FIC-0, FIC-1, or FIC-2), a synergistic, indifferent, or antagonistic interaction was found with a significant portion of the isolates (Table 2). For both the FIC-0 and the FIC-1, the median MIC (MIC$_{50}$) of 5FC for all isolates together decreased from 128 µg/ml when it was used alone to 4 µg/ml when it was used in combination with AMB. For the *A. terreus* isolates, the FIC-0 values were significantly higher and the FIC-1 values were significantly lower than those for the ITZ-S *A. fumigatus*, the ITZ-R *A. fumigatus*, and the *A. flavus* isolates. When the FIC-2 was used, the MIC$_{50}$ of 5FC for all isolates together decreased from 16 µg/ml when it was used alone to 1 µg/ml when it was used in combination with AMB. The FIC-2 values for the ITZ-R *A. fumigatus* and the *A. terreus* isolates were significantly lower than those for the *A. flavus* and the ITZ-S *A. fumigatus* isolates.

Greco model. The R^2s were above 0.90 in the majority of cases (median, 0.95; range, 0.58 to 0.98). Antagonism was found with 4.8%, indifference was found with 33.3%, and synergism was found with 61.9% of all 21 isolates. The ICα values for the *A. flavus* isolates were significantly lower than those for the ITZ-S *A. fumigatus*, the ITZ-R *A. fumigatus*, and the *A. terreus* isolates.

ITZ-5FC combination and the FIC index model. Depending on the definition of the FIC used (FIC-0, FIC-1, or FIC-2), a synergistic, indifferent, or antagonistic interaction was found with a significant portion of the isolates (Table 2). The differ-

interactions. This method is very sensitive, since it quantifies small amounts of fungal growth, and it is objective in contrast to visual reading of the turbidity, which makes the precise quantification of the hyphal growth of molds possible.

Another important reason for the different results and conclusions found in previous in vitro interaction studies might be the approach used to analyze the results. The most commonly used method is the calculation of the FIC index, but this approach has several major drawbacks. The first and principal problem in determining the FIC index is the choice of the MIC endpoints to be used for the two drugs alone and in combination (28). For antibiotics, complete inhibition of growth is used as the endpoint, and the difference between wells showing turbidity (no effect) and wells showing no turbidity (a maximum effect) is not more than 1 dilution. However, the concentration-effect relationship in MIC determinations for antifungals covers a range of dilutions, and the choice of the MIC endpoint is therefore more arbitrary. In addition, trailing effects are sometimes present. This problem especially arises when combinations of drugs with different MIC endpoints, such as the AMB-ITZ and AMB-5FC combinations, are used. For AMB, MIC-0 is taken as the endpoint, whereas MIC-1 or MIC-2 is taken as the endpoint for both ITZ and 5FC (23). When AMB is combined with ITZ or 5FC, the question arises of which MIC endpoint should be used: MIC-0, MIC-1, or MIC-2. In this study, the FIC index value found depended primarily on the MIC endpoint used for all three combinations (Fig. 1). In almost all cases, the differences between the FIC-0, the FIC-1, and the FIC-2 values were significant ($P < 0.05$). For all three combinations, the highest FIC index value was found with the FIC-0, followed by the FIC-1 and then by the FIC-2. Therefore, the weakest positive or the strongest negative interaction was found with the FIC-0, whereas the strongest positive or the weakest negative interaction was found with the FIC-2. For both the AMB-ITZ and the ITZ-5FC combinations, even the interaction found depended on the MIC endpoint used. When MIC-0 was used as the endpoint, antagonism was found; indifference was found when MIC-1 was used as the endpoint, and synergism was found when MIC-2 was used.

Another drawback of the FIC index model is the summary parameter and its interpretation. In this study, the ΣFIC_{min} was reported as the FIC index in all cases unless the ΣFIC_{max} was higher than 4; in these cases, the ΣFIC_{max} was reported as the FIC index. The ΣFIC_{max} was also reported as the FIC index for data sets where the ΣFIC_{max} was smaller than 4 but the ΣFIC_{min} was greater than 1. If for a data set the ΣFIC_{min} was lower than 0.5 and the ΣFIC_{max} was higher than 4, both the ΣFIC_{min} and the ΣFIC_{max} were reported. A problem of this approach is that one index (the FIC index), based on the results of one well, is used to describe the whole checkerboard; more refinement may be necessary, since at some concentrations there may be synergism but at others there may be indifference or even antagonism. A solution might be the calculation of the average FIC index and its 95% CI among all ΣFIC indices of a data set. Furthermore, outliers can affect the results.

Two approaches can be used to interpret the results of the FIC index model. The first approach looks at the results of each of the three replicates separately and determines the

interaction. For the interpretation of the FIC index, several different definitions have been described in the literature (1, 22, 24), but there is no consensus on which definition to use in interaction studies. In most cases, synergy was claimed when the FIC index was lower than or equal to 0.5, and antagonism was claimed when the FIC index was higher than 4. In all other cases, indifference was concluded. These cutoffs were chosen in order to diminish the effect of the intraexperimental error (± 1 dilution) of antifungal susceptibility testing, since a twofold dilution scheme was followed. In the approach that we used, the results of the three replicates were interpreted as one outcome (synergism, antagonism, or indifference), thereby constraining the interexperimental error. Thus, when the results of all three replicates were concordant, synergism or antagonism was claimed if the FIC indices were below 1 or above 1, respectively. In all other cases, indifference was concluded. An alternative approach would be to use the mean of the results from three replicates and the 95% CI; if 1 does not include the 95% CI, significant synergism or antagonism could be concluded. However, because the distribution of the FIC index is not normal, we preferred the approach mentioned.

Assessing the nature of drug interactions using the FIC index model faces several other problems besides the choice of the MIC endpoint, the lack of a good summary parameter, and the interpretation of this parameter, such as the difficulty in statistically interpreting the results and the imprecise approximation of the real FIC index when off-scale MICs are present (this situation was seen in this study for the ITZ-R *A. fumigatus* isolates with the combination of ITZ and 5FC).

Many of the above-mentioned problems of the FIC index can be overcome by using a response surface approach, which is fitted to the whole response surface. The use of a model fit makes an arbitrarily chosen endpoint unnecessary, allows the optimal use of information from the data, allows the determination of error estimates of the interaction coefficient and thereby indicates whether the interaction is significant or not, and allows an objective statistical criterion. Such a model was developed by Greco et al. (9) and has already been successfully used in the field of anticancer and antiviral drug interactions. It is a fully parametric model which is based on the assumption that the concentration-effect curves follow the E_{max} model (sigmoid curve with variable slopes) and which includes statistical significance levels of the interactions and summarizes them with a nonunit, concentration-independent interaction parameter.

There are also two approaches for the interpretation of results obtained with the Greco model. The first approach looks at the results of each of the three replicates separately and determines the interaction. If the estimate of ICα is positive and the 95% CI does not overlap 0, the interaction is significantly synergistic. If the estimate of ICα is negative and the 95% CI does not overlap 0, the interaction is significantly antagonistic. In all other cases, indifference is concluded. In the approach that we used, the results of all three replicates were considered as one outcome (synergism, antagonism, or indifference). Thus, when the results of all three replicates were concordant, synergism or antagonism was claimed if the estimate of the ICα was above or below 0, respectively. In all other cases, indifference was concluded.

In this study, differences were found between the FIC index

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model and the Greco model. These differences could have been caused by the drawbacks of the FIC index, which we already mentioned, but also by the drawbacks of the Greco model (19). The first problem is that the Greco model does not describe precisely the entire response surface, as statistically significant deviations from the model were found. This was more pronounced in combinations where trailing phenomena were apparent or with very strong synergistic interactions. In the first case, the E_{max} model does not adequately describe the data, while in the latter case a part of the problem is the heterogeneity of the data. In these cases, the levels of growth at each combination were overestimated by the Greco model compared with the growth that was observed. Another problem is that, because of the nature of the model, the estimates obtained are less reliable for antagonistic interactions. For these interactions, the Greco model tends to produce an estimate higher than the observed effect at high concentrations, which is an innate property of the model. The Greco model also provides only one ICα value to describe the interaction, but the assumption that only one interaction can take place when two drugs are combined is disputed because interactions in which both synergism and antagonism were present at different concentrations of the two drugs have been described (2, 10). However, to calculate this ICα value and thus determine the interaction, the model incorporates interaction data from all wells of the checkerboard. Finally, statistically significant results of the response surface fit do not necessarily indicate clinical significance. An ICα value ($\pm$ the 95% CI) of -0.03 (±0.01), for example, would indicate statistically significant antagonism. However, the clinical interpretation remains to be elucidated. As is true for all tests and their outcomes, test results must be related to the clinical outcome with respect to the interaction found, the side effects, the costs, etc. However, at least these decisions can be made by a test result that is more objective than that obtained by the FIC index.

In an earlier published study (28), we found that there was a correlation between the ICα values and the FIC indices. This was confirmed by J. Meletiadis et al. (unpublished data). To determine whether the value of the ICα could predict the magnitude of the FIC index, Meletiadis et al. analyzed the negative and positive ICα values with their corresponding FIC indices separately. A statistically significant correlation was found for the positive ICα values and the corresponding FIC indices, while for the negative ICα values and the corresponding FIC indices no statistically significant correlation was found. So, it seems that there is a correlation between the ICα values and the FIC indices when there is a positive interaction (synergism) but not when there is a negative interaction (antagonism), although the exact quantitative relationship is not clear. This can be due to either the FIC index or the Greco model.

Further investigation in order to find the best model for determining the in vitro interaction of antifungal drugs should be performed. However, we consider the limitations of the FIC index model, in particular the arbitrary results, to be far greater than those of the Greco model in characterizing antifungal drug interactions.

Although we found some differences between the results of the FIC index model and the Greco model and between the different *Aspergillus* species tested, the combination of AMB with 5FC was the most potent against *Aspergillus* species in vitro. Previous in vitro studies showed that this combination was mostly additive or synergistic against *Aspergillus* species (7, 13, 14, 16, 25). However, in one study antagonism was observed (5). These seemingly contradicting results may have been due to the methods used in those studies, the FIC index, or the interpretation thereof. It must be realized, however, that no clear correlation has been shown between the in vitro synergism of AMB and 5FC and the in vivo outcome of aspergillus infections. These are highly warranted.

We conclude that the use of the response surface approach for the interpretation of in vitro interaction studies of antifungals may be helpful in order to predict the nature and intensity of the drug interactions. However, the correlation of these results with clinical outcomes remains difficult and needs to be further investigated.

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